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PERSPECTIVES IN CLINICAL NEPHROLOGY

A reappraisal of immune-mediated glomerulosclerosis

The term glomerulosclerosis is generally used to designate glomerular scarring. It is a severe complication of many glomerular diseases and can lead to the total loss of renal function. Affected patients are dependent on life-long hemodialysis or require kidney transplantation. It is generally accepted that the pathogenesis of glomerulonephritis and glomerulosclerosis is complex, and that multiple events contribute to the ultimate sclerotic disruption of the glomerulus. The structure of the normal glomerulus can be injured by many mechanisms, which can be roughly divided into two groups, that is, immunologically mediated and non-immunologically mediated. Obviously, both forms of injury cannot be clearly separated since reduction in the number of functional nephrons as a result of immunologically mediated injury can result in hypertension in the remaining nephrons, thereby also contributing to damage of kidney tissue. Moreover, the proteinuric state can be nephrotoxic by itself and cause (further) damage leading to chronic inflammation in the kidney; however, cause and effect still need to be elucidated.

The initial event in the development of immunologically mediated glomerulonephritis is the formation of immune complexes in the glomerular mesangium and along the glomerular capillary wall. Damaged glomerular cells respond by producing cytokines including chemoattractants, and damaged matrix components can directly activate inflammatory cells. In most, but not all glomerulopathies complement activation is also involved. In response, an inflammatory reaction is initiated in the glomerulus. The recruited inflammatory cells produce cytokines such as transforming growth factor-β (TGFβ) and platelet-derived growth factor (PDGF), which alter the gene expression of resident glomerular cells, thereby triggering increased production and secretion of extracellular matrix (ECM) components and of other chemoattractants and activators which extend the pathogenic process. Altered expression of the ECM may not only include quantitative alterations. More and more evidence is appearing that qualitative alterations in matrix molecules such as alternative splicing of pre-mRNA, differentiated expression of individual subchains and neo-expression of epitopes occur as well.

In our laboratory intramolecular alterations of matrix molecules occurring during the development of glomerulosclerosis became a focus of interest and are the main topic of this review. Multiple models exist for experimental immune-mediated glomerular diseases. In this paper only two of them have been used to investigate the rather unexplored field of qualitative matrix alterations, that is, chronic graft-versus-host disease (GvHD) in the mouse and chronic serum sickness in the rat. These models were compared to determine whether differences in disease initiation result in different compositions of the glomerulosclerotic lesions. In this review these models and the inflammatory reaction leading to alterations in the matrix will be briefly introduced. Concomitantly, the quantitative and qualitative alterations of matrix molecules occurring during the development of glomerulonephritis and glomerulosclerosis in these and other models will be discussed. However, we should keep in mind that the nature of progression of disease may depend on the initiation of injury.

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Induction of experimental immune complex glomerulonephritis

Chronic GvHD in the mouse and chronic serum sickness in the rat are models for immune complex-mediated glomerulonephritis. GvHD is induced in (C57BL10 × DBA/2)F1 hybrid mice by injection of DBA/2-donor lymphocytes. A defect in anti-F1 T-cytotoxicity in the DBA/2 inoculate leads to the development of chronic autoimmunity in this specific strain combination. The recipient mice develop a variety of pathological alterations associated with the formation of autoantibodies. Laminin and the glomerular enzyme dipeptidylpeptidase type IV (gp90 = CD26) are prominent nephritogenic autoantigens in this model. Furthermore, autoantibodies against nucleosomal antigens may bind via histones to the glomerular basement membrane (GBM), particularly to heparan sulfate, and thus play a role in the induction of renal disease. The renal lesion in this model is a lupus type of glomerulonephritis, often accompanied by a severe nephrotic syndrome [1]. In chronic serum sickness in rats, the glomerulonephritis is thought to result from glomerular deposition of soluble immune complexes present in the circulation. Three discrete stages can be discriminated through which the disease progresses [2]. Immune complexes develop in the first, mild stage and, depending on their size, are deposited either in the glomerular capillary wall or in the mesangium. This interferes with the functioning of the GBM as a filtration barrier and accordingly leads to the onset of proteinuria, characteristic of the moderate stage. During the course of the disease, expansion of the mesangial matrix and thickening of the GBM occur, finally resulting in the development of glomerulosclerosis [3]. This disturbance of kidney function is detected only in the severe stage.

Immune complex deposition leads to inflammation and, via the release of cytokines, to alterations in the matrix

In both models the immune complexes are located along the glomerular capillary wall mainly subepithelially, but subendothelial deposits can be found as well. In chronic serum sickness fibrinogen is deposited along the endothelial cells at an early stage, possibly indicating damage to the endothelial lining. Early deposition of fibrinogen is not observed in chronic GvHD. In both models the glomerular damage initiated by the presence of immune complexes is accompanied by complement activation, the release of procoagulant, chemotactic and mitogenic cytokines by damaged cells [4–7], followed by an inflammatory reaction. The infiltrating inflammatory cells constitute a major source of a

variety of proteolytic enzymes, thereby contributing to glomerular injury [8–10], and may affect intrinsic glomerular cells via the production and secretion of cytokines [11–15]. Among them is the cytokine PDGF, which is a stimulator of mesangial proliferation and is, like epidermal growth factor (EGF), thought to be involved in the regulation of the glomerular matrix [16, 17]. However, the cytokine most notorious for its effect on ECM synthesis and turnover is TGFβ [18, 19]. This cytokine is a stimulator of ECM production, an inhibitor of ECM-degrading metalloproteinases and a stimulator of their natural regulators [18]. Indeed, using its natural inhibitor decorin or antibodies against TGFβ, the overproduction of glomerular matrix in anti-Thy-1.1 nephritis could be prevented. Also, neutralization of PDGF with anti-PDGF antibodies in the rat Thy-1.1 model of mesangioproliferative glomerulonephritis reduced not only mesangial cell proliferation but the expansion of the ECM as well [16]. Moreover, *in vivo* transfection of genes coding for TGFβ1 and PDGF-B into a rat kidney resulted in extensive ECM expansion and increased cellularity, respectively [20]. These findings strongly suggest that those cytokines may indeed be involved in the overproduction of matrix proteins *in vivo*. Resident glomerular cells contribute to extension of the pathogenic process by production of the same and other cytokines.

In most glomerulopathies inflammatory reactions which lead to increased ECM synthesis are not restricted to the glomerulus. Intraglomerular inflammation and cytokine release can also initiate an inflammatory reaction in the extraglomerular compartment, and thus mediate the genesis of interstitial fibrosis [21, 22].

Quantitative alterations in the ECM

Many glomerular diseases are associated with changes in the expression of ECM components. These may vary from very minor changes, such as in early membranous glomerulonephritis, to extensive alterations of the glomerular mesangial matrix and GBM as in metabolic diseases (diabetes, amyloidosis), hereditary diseases (Alport's syndrome) and immunologically mediated diseases (systemic lupus erythematosus, Goodpasture's syndrome, IgA nephropathy, etc.). Changes in ECM distribution patterns have been observed in human glomerular diseases [23–27] as well as in animal models [28–33]. Evidence for enhanced local matrix synthesis, as suggested by increased steady-state mRNA levels for individual ECM components, has been found in biopsies of diabetic patients [34] and in several experimental models for glomerulonephritis and glomerulosclerosis [29–31, 33, 35, 36]. In murine chronic GvHD and chronic serum sickness, steady-state mRNA levels for all ECM components examined have increased by an early stage in the disease [33, 36]. A few weeks later these raised levels are reflected in enhanced amounts of the ECM molecules in the glomerular matrix, which expands as a consequence [32, 33]. The time span between the elevation of mRNA steady-state levels and microscopical expansion of the glomerular matrix is relatively long (at least 2 weeks in chronic GvHD and approximately 15 weeks in chronic serum sickness). Since the ECM consists of a rather complex network of large molecules composed of various subchains, it may be hypothesized that the production, secretion and deposition of ECM molecules in a matrix are rather time consuming. In addition, changes in mRNA stability may not be ruled out as a cause of the increased mRNA levels. In most cases increased mRNA steady-state levels precede the morphological changes. This observation may be of importance in the early diagnosis and prevention of glomerular diseases in patients. Of course, what really counts is the protein product of the mRNA, and therefore it remains essential that histopathologic functional correlations are verified.

Matrix degradation

Accumulation of ECM components can be further enhanced by the ability of cytokines to suppress the expression of proteases and stimulate the expression of protease inhibitors. In some glomerular diseases changes in the expression of metalloproteinases and their inhibitors have been shown to contribute to altered matrix deposition [37, 38]. The role of the metalloproteinase, 72-kD type IV collagenase, was investigated in chronic GvHD and chronic serum sickness (unpublished results). In both, the expression of 72-kD type IV collagenase in the glomeruli did not change dramatically during the course of the disease. The mRNA levels in isolated glomeruli were only slightly increased (approximately 1.5- to 2-fold in the final stage of the disease) as compared with those in the glomeruli of normal animals. By histological staining using antibodies against collagenase type IV, fewer than two positive cells per glomerulus were detected in sections of both normal mouse and rat kidney tissue. The experimental groups did not manifest any significant change in the number of positive cells expressing this metalloproteinase at any time point. It may be difficult to demonstrate low levels of collagenase induction. Alternatively, 72-kD type IV collagenase mRNA may be present in cells and tissues in a translationally inactive form.

Qualitative alterations in matrix components

Alternative splicing of fibronectin

Not all of these changes of the matrix can be explained by an increase in the amounts of ECM components. Intramolecular changes may play an important role in the development of glomerulosclerosis as well. Splicing patterns for fibronectin have been shown to change in rats with anti-GBM glomerulonephritis or with anti-tubular BM-induced tubulointerstitial nephritis [39]. Therefore, in chronic GvHD and chronic serum sickness, post-transcriptional splicing patterns of mRNA coding for the V-region of fibronectin were investigated during the development of glomerulonephritis and glomerulosclerosis [40]. The ratios between the three fibronectin mRNA isoforms which result from alternative mRNA splicing were found to change towards increased exclusion of the V-region. As a consequence, specific domains containing sequences important for integrin binding are expressed relatively less. In chronic serum sickness this relative increase in the V^- isoform of fibronectin was observed during the whole course of the disease. However, in chronic GvHD these altered ratios were only observed two weeks after the first injection of parental lymphocytes, and then the splicing pattern returned to that seen in the glomeruli of normal adult mice. Stimulation of mouse mesangial cells by TGFβ did not change the splicing pattern of the fibronectin V-region. It may be speculated that TGFβ does not play a role in the induction of alternative splicing of the V-region of fibronectin in chronic GvHD and in chronic serum sickness.

Differentiated expression of collagen type IV subchains

A second ECM component exhibiting intramolecular changes which may contribute to malformations in the glomerular matrix

is collagen type IV. In our own studies with Northern and dot blot analysis, probes coding for the NC1 domains of $\alpha 3$, $\alpha 4$, and $\alpha 5$ minor chains of mouse collagen IV and for the traditional collagen IV major chains $\alpha 1$ and $\alpha 2$ were used to investigate the molecular composition of the glomerular matrix during the development of experimental glomerulosclerosis [41]. There was an earlier and more profound mRNA increase of the minor chains compared with that of the major chains. However, the chain which is preferentially expressed differs between isolated glomeruli and whole-kidney tissue, and between different experimental models. In isolated glomeruli mRNA expression was most abundant for $\alpha 3$ in GvHD, but for $\alpha 4$ in chronic serum sickness. In whole-kidney tissue mRNA for the $\alpha 5$ chain was most preferentially increased. Whether interspecies variation plays a role remains to be determined. These experiments, together with the *in vitro* studies of others [39], strongly suggest differential regulation of the individual collagen IV chains at the transcriptional level. Protein production, assembly and degradation of the individual chains are currently under investigation.

Differential expression of collagen IV subchains has been observed in other glomerulopathies as well. In diabetic nephropathy, the minor chains of collagen type IV are principal constituents of the thickened GBM, whereas the traditional chains are prominent within the expanded mesangium. However, with progression towards sclerosis, hyalinized glomeruli come to contain only minor chains [43]. Additionally, the appearance of $\alpha 3$ and $\alpha 4$(IV) collagen in spikes and $\alpha 1$ and $\alpha 2$(IV) collagen subendothelially in the thickened GBM was observed in membranous nephropathy [25]. Thus, changes in ratios between the individual collagen IV chains in disease states may lead to malformation of the collagen IV network and thereby exert an important effect on the structure and biological function of the glomerulus, and play a role in the development of glomerulosclerosis and renal dysfunction.

Neo-expression of laminin epitopes

The third molecule with qualitative changes which have been observed during the development of experimental glomerulonephritis and glomerulosclerosis is laminin. Laminin epitopes that are normally present in the GBM only during embryogenesis reappear in the glomerular capillary wall in a late stage of chronic GvHD [44]. Whether this is the result of local synthesis, redistribution or unmasking of epitopes remains to be determined. A change in the expression of laminin isoforms has also been observed in other systems, for example, after insulin treatment of rat mesangial cells in culture [45].

Taken together, increasing evidence is emerging that intramolecular changes in ECM components may represent another important mechanism involved in the development of glomerulosclerosis.

Development of end-stage glomerular sclerotic lesions

At a certain time point in murine chronic GvHD and chronic serum sickness, the expansion of the mesangial matrix and thickening of the GBM are followed by the development of glomerular sclerotic lesions. In both models glomerulosclerosis is first focal and segmental; however, in chronic GvHD it becomes diffuse and global at the end of the observation period. Strikingly, abundant amounts of fibronectin were present in the end-stage sclerotic lesions in both models [32, 33, 46]. Other ECM components like laminin and several collagen types were present only in the remnants of the mesangial matrix and GBM in the periphery of these lesions. A specific accumulation of plasma-fibronectin from the circulation was found to lead to the presence of fibronectin in the end-stage renal lesions [46]. Nonspecific trapping of constituents from the circulation does not play a significant role in the development of glomerulosclerosis in our models, since albumin, fatty acids and transferrin were not observed in the end-stage lesions. Thus, despite the differences in disease initiation, the development and composition of the end-stage glomerulosclerotic lesions seem to be similar in chronic GvHD in mice and chronic serum sickness in rats.

As already indicated above, simultaneously with the development of glomerulosclerosis, a second inflammatory reaction takes place in the tubulointerstitial compartment of the kidney surrounding the damaged glomeruli. Twelve weeks after the induction of chronic GvHD, interstitial infiltrates of CD3-positive cells are present. *In situ* hybridization experiments showed increased ECM mRNA expression in the perivascular and periglomerular regions and in the interstitium, possibly contributing to the development of interstitial fibrosis [33, 47]. The cells which show increased expression of ECM mRNA have not been identified yet, but a vascular adventitial cell (VAC) has been put forward as a likely candidate [48]. Furthermore, although the disease related to chronic interstitial nephritis may have much the same appearance as the interstitial nephritis related to glomerulonephritis, there may be significant differences yet to be elucidated.

In our models glomerulosclerosis is accompanied by local activation of the blood coagulation system. Fibrin deposits can occlude the glomerular capillaries. Furthermore, components of the coagulation cascade can contribute to glomerular damage via the attraction of monocytes and/or macrophages [49] and a direct cytotoxic effect on mesangial cells [50]. The accumulation of fibronectin in glomeruli observed in chronic GvHD and in serum sickness may be secondary to triggering of the coagulation cascade. However, the mechanism of binding of fibronectin in the end-stage sclerotic lesions remains unclear. In order to investigate the role of coagulation in the development of glomerulosclerosis, accumulation experiments were performed in the presence of the anti-coagulant heparin (Bergijk et al, unpublished results). Addition of heparin to fluorescein isothiocyanate (FITC)-conjugated plasma-fibronectin before injection into a GvHD week 12 mouse blocked the accumulation of plasma-fibronectin-FITC in a dose-dependent manner. Furthermore, plasma-fibronectin accumulation could also be blocked by using *N*-desulfated, non-anti-coagulant heparin. This suggests that the blocking effect of heparin is achieved via direct interference in the interaction between plasma-fibronectin and glomerular structures and not by its anti-coagulant effect.

Fibronectin contains domains which are specifically recognized by receptors, such as integrins, on the cell surface [51]. The expression of these integrins as well as their affinity for their respective ligands change in the course of glomerular disease [51–53]. Thus, altered expression or function of integrins may well lead to enhanced binding of plasma-fibronectin in our models. Several members of the $\beta 1$ integrin subfamily, that is, $\alpha 3$, $\alpha 4$, $\alpha 5$ and αv, as well as the integrin $\alpha IIb\beta 3$ are known to bind fibronectin [51]. However, hybridization of RNA extracted from pooled, isolated glomeruli at different time points in chronic GvHD with cDNA probes against the $\alpha 3$ and $\alpha 4$ integrin chains

did not show statistically significant changes in mRNA expression. Glomerular mRNA steady-state levels for the $\alpha 6$ integrin chain, which specifically binds to laminin, increased four weeks after induction of chronic GvHD and remained at this high expression level during the course of the disease. In normal human glomeruli $\alpha 6\beta 1$ is expressed on glomerular epithelial podocytes only during glomerular development. The increased $\alpha 6$ mRNA steady-state levels in diseased glomeruli suggest a re-expression of this receptor, which may possibly play a role in the abnormal accumulation of laminin and the reappearance of 'immature' laminin epitopes [44]. These data suggest that integrins play a role in the alteration of the glomerular matrix, but that they are probably not involved in the trapping of plasma-fibronectin.

Another receptor known to bind fibronectin is CD44 [54]. Recently, cultured human mesangial cells and mesangial cells in the proliferative phase of the rat α-Thy-1.1 model for membranoproliferative glomerulonephritis were found to express CD44 on their surface [55]. However, this receptor is not expressed in glomeruli after the induction of chronic GvHD and chronic serum sickness, and therefore does not play a role in trapping plasma-fibronectin from the circulation. Moreover, fibronectin has an affinity for immunoglobulins, which are present in the diseased glomeruli in the models discussed here [56].

Up to this point we know that the specific accumulation of plasma-fibronectin from the circulation in the end-stage sclerotic lesions in chronic GvHD and serum sickness cannot be explained by either increased *de novo* synthesis or decreased degradation of fibronectin, nor by increased expression of adhesion molecules of the integrin family. In addition to its ability to bind to receptors, the fibronectin molecule can bind to a number of ECM components, including collagen and heparan sulfate proteoglycan [57]. Accumulation of plasma-fibronectin in the end-stage sclerotic lesions in our models may also be initiated by binding of fibronectin to other ECM components. In Heymann nephritis enhanced binding of fibronectin to the glomerular capillary wall is thought to be due to an increase in collagen in the GBM and/or denaturation of collagen type IV [58]. Intramolecular changes of ECM molecules resulting from several possible mechanisms including alternative splicing of pre-mRNA may also be involved in trapping exogenous plasma-fibronectin. Since the rate of incorporation of V^+V^+ homodimeric fibronectin into fibrin clots is significantly slower than that of heterodimeric V^+V^- fibronectin [59], increased amounts of the latter isoform may facilitate the accumulation of plasma-fibronectin from the circulation into the end-stage sclerotic lesions. Therefore, the shift towards a plasma-fibronectin-like isoform of fibronectin in these models may possibly contribute to the development of glomerulosclerotic lesions in the final stages in these diseases. Moreover, unbalanced differential expression of subchains and/or neo-expression of specific epitopes of ECM components in the glomerulus may also disturb normal matrix interactions.

Clinical implications

Insight into the pathogenetic pathways of experimental and human glomerulonephritis and glomerulosclerosis opens the way to improved early diagnosis and preventive or therapeutic intervention. Interference with the inflammatory reaction may be established via the blockage of adhesion molecules mediating the influx, adhesion, and migration of inflammatory cells [60]. Modulation of the cytokine network by monoclonal antibodies directed against certain cytokines may reduce glomerular damage as well [61, 62]. In addition to interference with the early inflammatory reaction, there may be situations which require controlling cytokines which regulate the matrix homeostasis. In this respect, it was postulated that antibodies against both TGFβ and PDGF as well as the natural inhibitor of TGFβ, decorin, would be able to prevent the development of experimental glomerulonephritis [16, 18]. Recently, we showed that the development of glomerulonephritis and glomerulosclerosis in chronic GvHD was prevented by early cyclosporine A (CsA) treatment [47]. There was no interference by this treatment with the induction of the GvHD itself, since autoantibody levels in sera and deposition of immunoglobulins in the kidneys did not change in these mice compared with untreated GvHD mice. CsA administration had to be started prior to the onset of proteinuria. In a clinical situation, in which proteinuria is used as a diagnostic parameter, one is often too late with treatment as far as glomerulosclerosis is concerned.

In our experiments, mice with chronic GvHD show increased mRNA levels of ECM components in their kidneys even before signs of proteinuria arise. This stresses the diagnostic value of early mRNA analysis in slowly progressive glomerulonephritis, which has also been suggested by Dr. L. Striker [34]. The very sensitive technique of competitive rtPCR will provide an even more useful tool in mRNA analysis of small tissue samples like human biopsies.

Since hemodynamic factors can play a role in glomerular damage either early in the disease as initiators of the whole pathogenic cascade or later as a result of glomerular obstruction, reduction of hypertension is important as well, as reviewed elsewhere [63]. This can be accomplished by diverse methods, such as dietary protein restriction, angiotensin converting enzyme inhibition, and lowering of lipids [64].

Finally, since patients are often hospitalized when the morphological signs of matrix accumulation have already become established, it is necessary to design therapeutic strategies in order to prevent further damage and the development of glomerulosclerosis. The data reviewed here reveal that the end-stage sclerotic lesions in the chronic GvHD and chronic serum sickness models result from a specific accumulation of plasma-fibronectin, possibly complicated by factors of the coagulation cascade. Also, in human glomerulonephritis and glomerulosclerosis, altered fibronectin expression was often observed [65, 66]. Therefore, means to interfere with either the specific accumulation of fibronectin or the formation of fibrin fibers in the lesions are desirable. As already indicated above, in addition to its anti-proliferative effect [67], recent data suggest that heparin could be a useful tool in the prevention of plasma-fibronectin accumulation in the end-stage sclerotic lesions. Furthermore, synthetic peptides may assist by blocking receptors or other glomerular structures which bind fibronectin.

Hypothetical pathogenesis of immunologically mediated glomerulosclerosis

In this review, the role of the ECM in the development of experimental immunologically-mediated glomerulosclerosis was discussed. Based upon our own observations, those of others and partly on speculation, a hypothetical pathogenetic pathway concerning the development of glomerulosclerosis in immunologically mediated renal diseases emerges. This hypothesis is summarized in Figure 1. However, in extrapolating to all models of

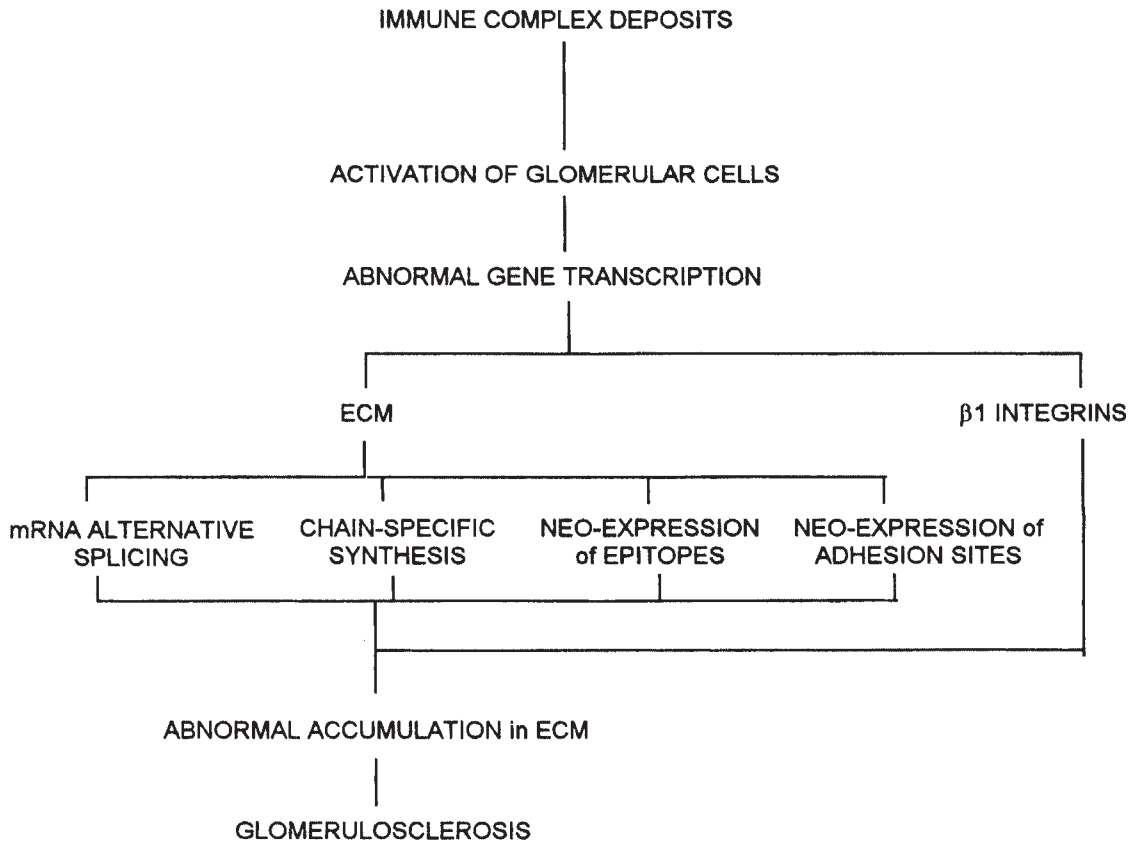

Fig. 1. *Simplified schematic representation of the processes leading to glomerulosclerosis.*

autoimmunity one should always keep in mind that differences in chronologic appearance, relative quantity, and dynamic interplay of cytokines probably play a role in the phenotypic end expression.

The initial event in the development of immunologically mediated glomerulonephritis is glomerular injury caused by the formation of immune complexes in the glomerular mesangium and along the GBM. Damaged matrix components and, via the release of cytokines, damaged glomerular cells activate inflammatory cells. In response, an inflammatory reaction is initiated in the glomerulus. The recruited inflammatory cells produce cytokines such as TGFβ and PDGF, which alter the gene expression of resident glomerular cells, thereby triggering increased production and secretion of ECM components, and of paracrine and autocrine cytokines. These chemoattractants and activators augment the pathogenic process. How the increased transcription of genes coding for ECM components and cytokines is triggered is currently the topic of many investigations. In recent years, *cis*- and *trans*-acting elements have been shown to regulate: the production of collagen type IV [68, 69], collagen type I [70], and laminin [71]; secretion and alternative splicing of fibronectin [72–74]; and production of cytokines such as TGFβ [75]. Triggering of the intrinsic glomerular cells leads not only to quantitative changes in the expression of ECM components, but probably to qualitative changes as well. This, possibly together with altered expression and/or affinity of receptors, may facilitate the accumulation of plasma-fibronectin, resulting in the development of the end-stage sclerotic lesions. Moreover, factors produced by glomerular cells may diffuse through Bowman's capsule and/or through the efferent vasculature to the interstitium, where they can initiate interstitial fibrosis. Further investigations are needed to unravel the complex pathogenesis of glomerulosclerosis, thereby leading to improvement of strategies in the early diagnosis and treatment of patients with renal disease.

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